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Abstract: Cysts of *Chattonella antiqua* (Raphidophyceae) were obtained in laboratory cultures for the first time. They were formed on the surface of glass coverslips added to both P- and N- depleted mediums. The yield was 50–250 cysts cm^{-2}. Crossing experiments showed the highest cyst production in mixed cultures, although cysts were observed in clonal cultures as well. Cysts had approximately double the DNA content of synchronously growing vegetative cells (G_1 phase). The encystment process was also monitored: Under P-depleted conditions, 'small cells' were formed that fused to become a 'triangle'-shaped cell which in turn changed into a cyst.

These results strongly suggest that cysts are the products of fusion and are diploid. 'Small cells', 'triangle'-shaped cells, and cysts are considered to be gametes, planozygotes, and hypnozygotes, respectively. Significant mortality was observed in laboratory cysts stored in the cold and dark for five months, although those remaining were capable of germination to reestablish a motile, vegetative population.

1. Introduction

Life cycle changes that allow populations of some red-tide flagellates to alternate between a benthic and a planktonic existence clearly play important roles in the initiation and decline of blooms (*e.g.* Wall, 1975; Anderson and Wall, 1978; Anderson *et al.*, 1983). In the case of *Chattonella antiqua*, a raphidophycean flagellate which causes severe red tides during summer in the Seto Inland Sea, Japan (Ono and Takano, 1980; Nakamura *et al.*, 1989), benthic cells of this species (hereafter termed cysts) have recently been isolated from bottom sediments (Imai and Itoh, 1986, 1988) and the importance of these cysts as an inoculum to initiate red tides was suggested (Imai *et al.*, 1986).

However, the cysts of *C. antiqua* have not yet been observed in laboratory cultures, and thus little is known about the details of the encystment process. There is a clear need to understand the encystment/excystment process as a function of environmental parameters (*e.g.* Anderson and Wall, 1978; Anderson *et al*,. 1984; Binder and Anderson, 1987) and to assess the ecological roles of cysts in the population dynamics of *C. antiqua* more quantitatively. The first objective of this study was thus to form cysts of *C. antiqua* in laboratory cultures. After encystment had been achieved, experiments were conducted to determine whether the cysts were formed through a sexual process.

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2. Materials and methods

2.1. Organisms and culture conditions

Four strains of *Chattonella antiqua* (Ho-1, A-0, B-5, B-20) were used throughout the experiments. All strains were isolated from Harima-Nada, the Seto Inland Sea, and were clonal. Ho-1 was axenic and the others were not. All experiments were conducted using a modification of the TH-medium (Table 1). Maintenance and inoculum cultures were grown in the TH-medium. Since nutrient limitation is often effective in inducing encystment (*e.g.* Pfiester, 1975; Anderson *et al.*, 1984), the N, P-reduced TH-medium (=encystment medium) was used throughout the experiments (Table 1). In this medium, phosphorus limits the final yield of *C. antiqua* (Naka-

Table 1. Composition of the medium.

	TH-medium[d]	Encysment medium[d]
Filtered seawater[a]	950 ml	950 ml
Distilled water	50 ml	50 ml
NO_3^-	450 μM	30 μM
PO_4^-	30 μM	1 μM
$Si(OH)_4$	35 μM	20 μM
N1-metals[b]	30 ml	30 ml
Vitamin B_{12}	200 ng·l^{-1}	10 ng·l^{-1}
Thiamine	100 μg·l^{-1}	100 μg·l^{-1}
Biotin	100 ng·l^{-1}	100 ng·l^{-1}
TRIS	1 g	1 g
pH[c]	8.0	8.0

[a] Surface seawater from the Kuroshio area (*ca.* 35 ‰) was used.

[b] 1,000 ml of N1-metals contain: Na_2 EDTA·$2H_2O$ 1.0 g, $FeCl_3$·$6H_2O$ 63 mg, $CoSO_4$·$7H_2O$ 0.9 mg, $ZnSO_4$·$7H_2O$ 12 mg, $MnCl_2$·$4H_2O$ 32 mg, $CuSO_4$·$5H_2O$ 0.2 mg, Na_2MoO_4·$2H_2O$ 0.2 mg.

[c] pH of the medium was adjusted by the addition of HCl.

[d] These media were sterilized by autoclaving (120°C, 20 min).

mura and Watanabe, 1983). The strains ($\sim 2\times 10^4$ cells ml^{-1}) were inoculated into the encystment medium individually (1 ml l^{-1}) or in combinations (0.5 ml l^{-1} of each strain) and cultured at 25°C, with 150 μE m^{-2} sec^{-1} illumination on a 12:12 LD photoperiod (except for Experiment 5 which used a 14:10 LD). Encystment experiments were conducted when the above cultures reached the growth maximum ($2\text{–}3\times 10^3$ cells ml^{-1}; P-depleted).

2.2. General method for encystment

Since cysts of *C. antiqua* recovered from the field were often found on diatom frustules (Imai and Itoh, 1988), we hypothesized that cysts had a tendency to adhere to the surface of solid materials. Four pieces of coverslip (15 mmϕ, 1.8 cm^2, made of borosilicate glass) were placed in a 200-ml Erlenmyer flask with 100 ml of distilled water and autoclaved. The distilled water was then discarded and replaced with a culture of *C. antiqua* (100 ml) at its growth maximum. The culture was incubated under the conditions described above for two days and then placed in darkness (25°C) for eight days. At the end of that interval, each piece of coverslip was taken from the culture and rinsed in filtered seawater three times to remove motile cells. Then the surface was observed under a microscope, and the number of cysts adhering to the coverslip was enumerated.

2.3. Establishment of encystment

Experiment 1: A mixed culture of the four strains at the growth maximum (1 *l*) was divided into eight subcultures and incubated as described above with coverslips. After dark incubation had been completed, the surface of the coverslips were observed.

Experiment 2 (Time course of encystment): A multi-flask approach was used (Anderson and Lindquist, 1985). A mixed culture of the four strains at the growth maximum (2 *l*) was divided into 20 subcultures. During the period when subcultures were in continuous darkness, two or three subcultures were removed daily and the number of cysts on the surface of the coverslips was counted to monitor the time course of encystment.

Experiment 3 (Irradiance effects): A mixed culture of the four strains at the growth maximum (1 *l*) was divided into eight subcultures with coverslips. Half of the subcultures were incubated at 25°C, *ca.* 150 μE m^{-2} sec^{-1} under a 12:12 LD photoperiod for ten days, and the others were placed under conditions described in Section 2.2. At the end of the incubation, cysts were enumerated and a comparison was made between the effects of these two treatments.

2.4. Sexuality of cysts

Experiment 4 (Interclonal crosses): Using clonal cultures of the four strains at growth maxima (500 ml for each strain), interclonal crosses were examined. Experiments were conducted under all possible combinations of the clone: cultures of each strain (50 ml) were combined with itself and with each of the others (50 ml) in separate 200-ml Erlenmyer flasks. Duplicate flasks were used for each combination. Internal crosses between the strain A-0 and B-20 were reexamined. Experimental procedures were the same as those described above.

Experiment 5 (Comparison of DNA content between cysts and vegetative cells): Cysts for DNA measurements were obtained using a mixed culture of strains A-0 and B-20. Procedures fof fixation and DNA measurement are those or Cetta and Anderson (1990). Each piece of coverslip was rinsed in filtered seawater three times, fixed in 2% glutaraldehyde in filtered seawater

for *ca.* 10 min at room temperature and then stored at 4°C. The DNA content was measured within three weeks. Vegetative cells were obtained from a clonal culture of A-0 and B-20 in the TH-medium. Each strain was cultured in a 1-*l* Erlenmyer flask with a 500-ml medium under a 14:10 LD cycle (lights on at 06:30 and off at 20:30). When the cell concentration reached 500 cells ml^{-1} (mid-exponential phase), each culture was divided into 13 subcultures in 50-ml culture tubes (culture volume=25 ml), and incubated as described above. On the following day, one culture tube from each strain was sampled at 2 hr intervals. Five-ml subsamples were added to 2.5 ml of a 6% glutaraldehyde solution in filtered seawater in a 15-ml tube, fixed for *ca.* 10 min at room temperature and then stored at 4°C. The DNA content was measured within four weeks. Cell concentrations were also monitored by microscope counts using a 1-ml Sedgewick-Rafter chamber. Each sample is denoted by the number of hours into the light (L) or dark (D) period (*i.e.* 4L=four hours after the lights went on).

Staining of cysts and vegetative cells for DNA quantitation was conducted at room temperature (*ca.* 20°C) (Cetta and Anderson, 1990). Each piece of coverslip with fixed cysts was immersed in a Hoechst 33342 solution in filtered seawater (2 $\mu g\ ml^{-1}$) for ten minutes, rinsed two times in filtered seawater and placed in filtered seawater for DNA measurements, which were completed within four hours of staining.

Fixed samples of vegetative cells were concentrated by centrifugation in a 15-ml tube. All but 0.5 ml of the supernatant was removed by aspiration and the pellet was resuspended. Then, 0.5-ml of the Hoechst solution in filtered seawater (4 $\mu g\ ml^{-1}$) was added to the cell suspension, which was stained for 10 min. Cells were then rinsed three times in filtered seawater through a centrifuge and were used for DNA measurements, which were completed within 6 hours of staining.

DNA-Hoechst fluorescence was measured using a Zeiss IM35 inverted epifluorescent microscope (Zeiss filter set 48 77 02 plus red attenuating filter BG 38) with a 50 W mercury lamp: The microscope was equipped with a Nikon photometer system (P1). Each piece of coverslip with cysts or stained vegetative cells suspended in filtered seawater was placed in a Palmer-Maloney counting chamber. Samples were scanned under a tungsten light at 250 X, and individual cells were selected for measurement aligned in the center of the field. The light path to the photomultiplier was restricted by a pinhole large enough to admit light from only the selected nucleus. A switch-activated electronic shuttering system blocked the tungsten light source, briefly opened the mercury light path and the photometer light path for 800 ms, and the fluorescence reading was recorded. Autofluorescence of chlorophylls was eliminated by the red attenuating filter (BG 38). The blank value was measured on the area having no cells and was subtracted from the recorded value. DNA-Hoechst fluorescence intensity was measured for 156 cysts and 100 vegetative cells from each sample, expressed in arbitrary fluorescence units (AFU). Stability of the instrument was confirmed by checking against permanently-mounted dansyl chloride-stained plastic beads. Other experimental procedures were the same as those described by Cetta and Anderson (1990).

Experiment 6 (Observation of the encystment process): A mixed culture of A-0 and B-20 at the growth maximum (2 *l*) was divided into 20 subcultures. After the subcultures had been placed in continuous darkness, three or four subcultures were taken daily and observed using a 1-ml Sedgewick-Rafter chamber under an inverted microscope. When an 'unusual' cell (such as a fusing pair) was found, it was isolated using a Pasteur pipette and was placed into a well of a tissue culture plate (Sumitomo, 96 wells) with the N, P-omitted TH-medium (0.25 ml). Isolated cells were placed in continuous darkness (25°C) and were observed daily under an inverted microscope.

2.5. Cyst germination (Experiment 7)

Cysts obtained from Experiment 1 were used for this experiment. After counting the cysts, each piece of coverslip was placed in a glass vial (10 ml) with a 5-ml fresh encystment medium. The glass vial was then placed in continuous darkness and the temperature was decreased from 25 to 10°C at a rate of 2.5°C d^{-1}. Following storage at 9-10°C for five months, the temperature was increased to 22°C (optimum for germination; Imai *et al.*, 1984) at a rate of 2.5°C d^{-1}. Cysts were then incubated at 22°C,

70 μE m^{-2} sec^{-1} under a 12:12 LD photoperiod for seven days. After that, two ml of the medium in the glass vial was sampled and observed under a microscope to search for vegetative cells. In this experiment, 29 pieces of coverslip were used.

3. Results

3.1. Methods for encystment

When P-depleted mixed cultures of the four strains were placed in continuous darkness (Experiment 1), vegetative cells could easily be differentiated from non-motile cells which were formed

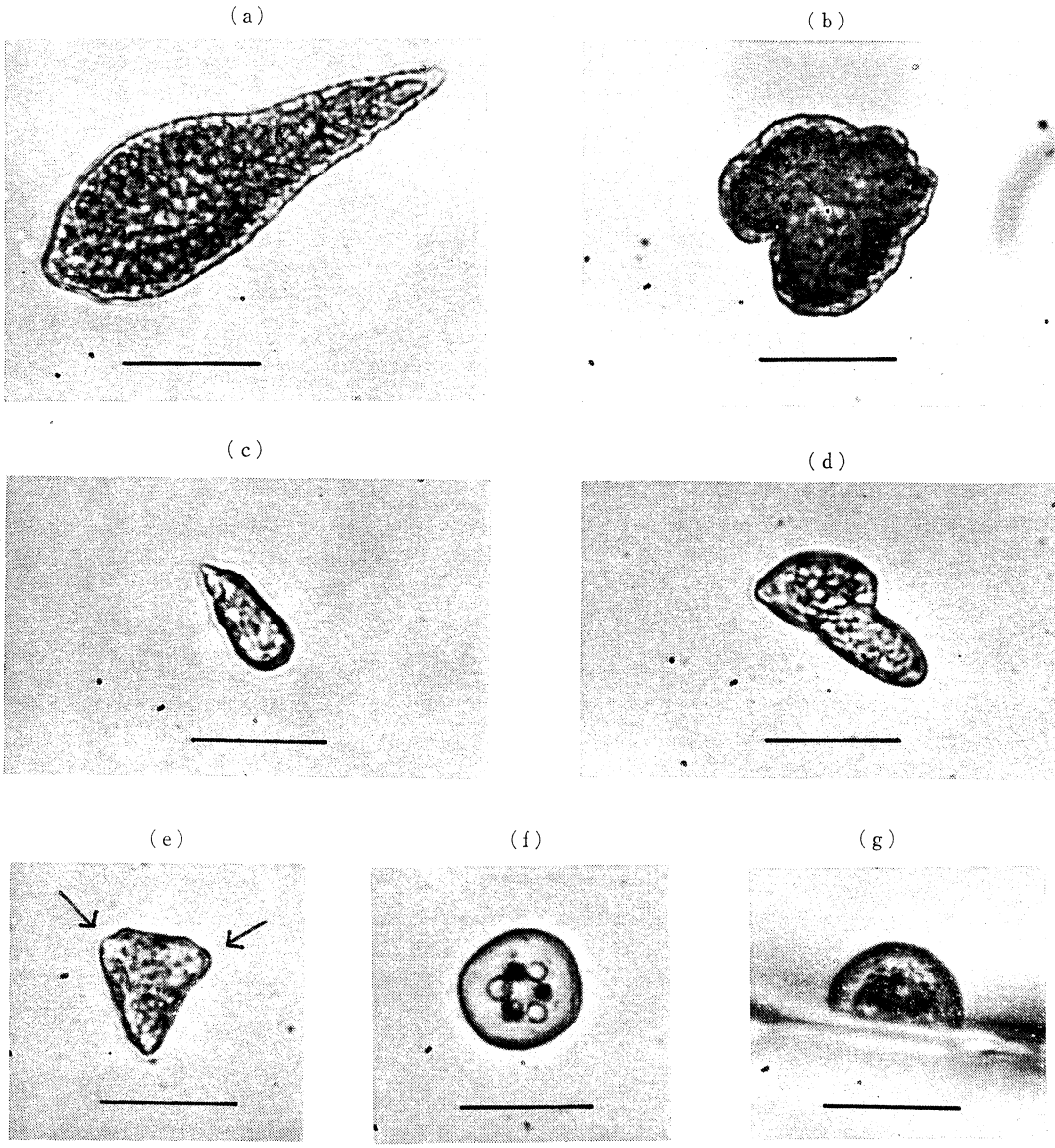


Fig. 1. *Chattonella antiqua* life history diagram. (a) Vegetative cell of *C. antiqua* in the exponential growth phase. (b) Dividing cell. (c) 'Small cell' of *C. antiqua* from P- depleted culture. (d) Fusing 'small' cells. (e) 'Triangle'-shaped cell (arrows indicate the position of flagella). (f) Laboratory cyst of *C. antiqua* adhering to the surface of coverslip (top view). (g) Laboratory cyst of *C. antiqua* adhering to the edge of coverslip (side view). Scale bar: 30 μm.

on the surface of coverslips at 50–250 cells cm^{-2} (Fig. 1 (f), (g)). These non-motile cells: (1) were mostly hemispherical, diameter 25–32 μm; (2) adhered to solid surfaces; (3) were yellow-greenish to brownish; and (4) contained several spots of a dark-brown or black material. These characteristics are consistent with those of cysts of *C. antiqua* recovered from the field (Imai and Itoh, 1988). Hereafter, these are termed laboratory cysts. Laboratory cysts were also formed at 30–170 cells cm^{-2} using N-depleted mixed cultures of the four strains (initial concentrations of NO_3^- and PO_4^{3-} were 10 and 3 μM, respectively).

Laboratory cysts were most numerous between days 2 and 3 (day 0=beginning of continuous darkness). After this period, the yield did not increase to any significant extent (Experiment 2; Fig. 2). Results from Experiment 3 indicate that laboratory cysts were also formed under a 12:12 LD photoperiod (Table 2), although the yield was much lower than that under continuous darkness.

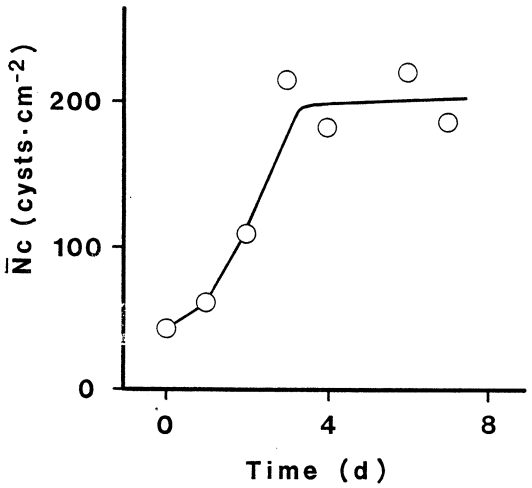


Fig. 2. Time course of *Chattonella antiqua* encystment. $\bar{N}_c$, Average cyst yield on coverslips (n=8 or 12). At t=0, cultures were placed in continuous darkness. SD of N_c was *ca.* 50% of $\bar{N}_c$.

Table 2. Effects of light conditions on encystment of *Chattonella antiqua*.

Condition	Nc[a)]	SD	n[b)]
12:12 LD	3	3	16
Continuous darkness	55	17	16

[a)] Averaged cyst yield formed on the surface of coverslip (cells cm^{-2}).

[b)] The number of the pieces of coverslip counted.

3.2. *Sexuality of laboratory cysts*

In order to determine the most effective combination of the four *C. antiqua* strains for encystment and to obtain preliminary information on whether laboratory cysts are sexual products, crossing experiments were conducted (Experiment 4, Table 3(a)). Strains Ho-1 and B-5 did not form cysts under the experimental conditions used, but A-0 and B-20 did. The yield from

Table 3. Averaged yield of *Chattonella antiqua* laboratory cysts from interclonal crosses (cells cm^{-2}).

(a)

	Ho-1	B-5	A-0	B-20
Ho-1	0	0	7(4)	58(25)
B-5		0	6(2)	10(5)
A-0			18(6)	238(89)
B-20				50(16)

(b)

	A-0	B-20
A-0	54(50)	168(78)
B-20		4(2)

* Figures in parentheses are the SD of cyst yield (n=8 for (a) and 24 for (b)).

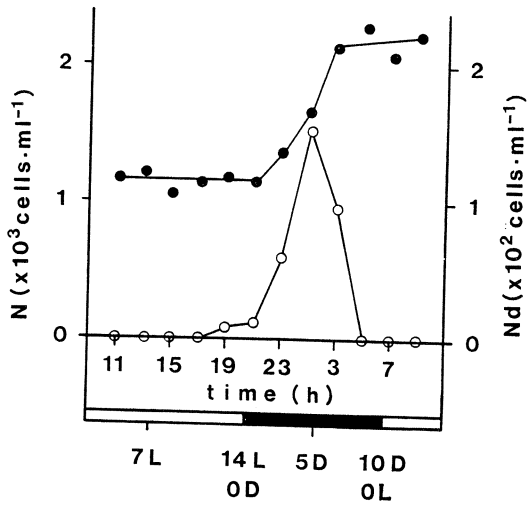
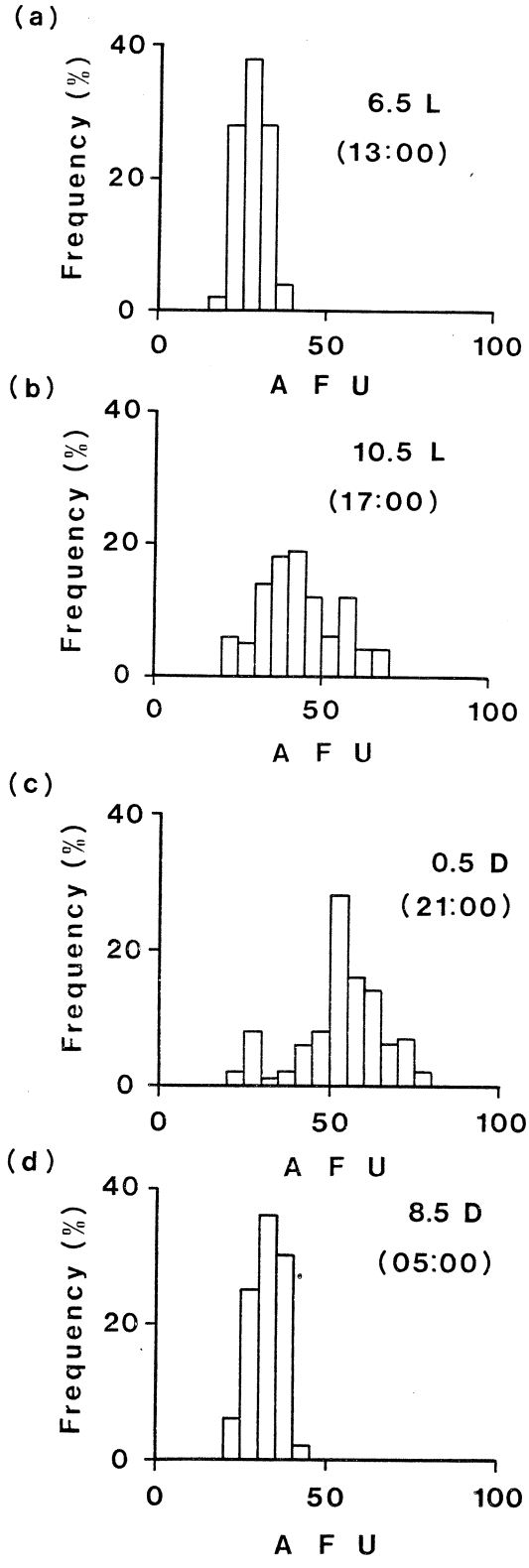


Fig. 3. Changes in the concentration of vegetative cells (N; ●) and dividing cells (Nd: ○) in synchronously growing *Chattonella antiqua* (B-20) under a 14:10 LD photoperiod.



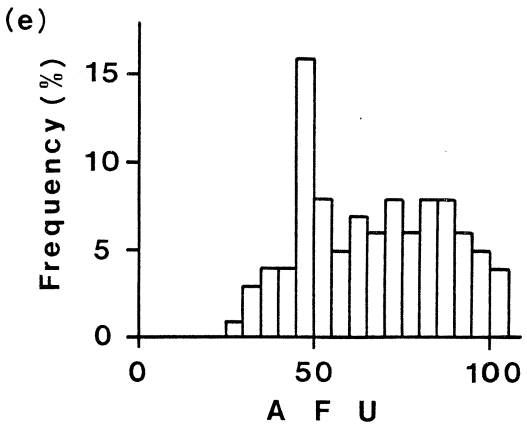


Fig. 4. Representative DNA fluorescence (AFU =arbitrary fluorescence unit) *vs.* frequency histograms in synchronously growing *Chattonella antiqua* (strain B-20) at 6.5 L(a), 10.5 L(b), 0.5 D(c), and 8.5 D(d). (e) DNA fluorescence (AFU) *vs.* frequency histograms in laboratory cysts.

the combination of A-0 and B-20 (238 cells cm^{-2}) was significantly higher than that for each clone individually (18 or 50 cells cm^{-2}). Interclonal crosses between A-0 and B-20 were reexamined (Table 3(b)) and the above results were shown to be reproducible.

The DNA content of laboratory cysts was measured and compared with that of vegetative cells as one way to ascertain whether sexuality had occurred (Experiment 5). Vegetative cell growth was synchronous and cell division mainly occurred in the dark between 2.5 D and 6.5 D; the cell concentration doubled during this period (Fig. 3). Representative DNA *vs.* frequency histograms of the vegetative cells of B-20 are shown in Fig. 4. At 6.5 L, all cells were in G_1 with one complement of DNA (Fig. 4(a)). At 10.5 L, replication of DNA began (Fig. 4(b)) and at 0.5 D, most cells were in G_2 with two complements of DNA (Fig. 4(c)). Then, at 8.5 D, when cell division had been completed, all cells were in G_1 once again (Fig. 4(d)). Similar results were obtained using an exponentially-growing culture of A-0 (data not shown). These results are consistent with another cell cycle study of *C. antiqua* obtained with a similar microscope photometer system with DAPI staining (Nemoto *et al.*, 1987). Cysts were uninucleate and their DNA histogram is shown in Fig. 4(e). DNA

content of the cysts is apparently higher than that of G_1 cells (Fig. 4 (a), (d)) and the mean (±SD) DNA content of the cysts (67±(20) AFU) is approximately double that of G_1 cells (33±(7) AFU). The DNA histogram of cysts is broader than that of vegetative cells, and the broader distribution of cyst-DNA can be due to the presence of 1C, 3C or 4C cells. However, the most likely explanation is that the cysts are 2C, the broader distribution reflecting difficulties in measuring morphologically-different cysts.

In order to observe the encystment process directly, Experiment 6 was conducted. When the mixed culture of A-0 and B-20 reached its growth maximum, a significant portion of the population (*ca.* 30% of the total cells) was very small in size (25-35 μm; Fig. 1(c)) relative to exponentially growing cells (*ca.* 100 μm; Fig. 1(a)). Our preliminary experiments showed that the more 'small cells' formed, the higher the yield of laboratory cysts. After the cultures had been placed in continuous darkness, 'fusing' pairs of the 'small cells' were often observed (Fig. 1(d)). They were asymmetrically shaped and easily distinguishable from dividing cells (Fig. 1(b)). 'Triangle'-shaped cells (Fig. 1(e)) were also observed during this period. Two flagella, originating at different corners of the triangle, were observed. We isolated 107 pairs of 'fusing' cells: 40 pairs changed to 'triangle'-shaped cells within 1-3 days; among newly formed 'triangle' cells, 24 became laboratory cysts, which adhered to the bottom of the well. We also isolated 29 'triangle'-shaped cells, 14 of which changed to laboratory cysts within 1-5 days. The isolates of 'fusing' pairs or 'triangle'-shaped cells, which did not change into 'triangle' or laboratory cysts, either died or in some cases the former was divided again into two 'small' cells. The above observations, together with the results that the mean DNA content of cysts was approximately double that of G_1 cells, strongly suggest that cysts are the product of fusion and are diploid.

3.3. Cyst survival and germination

Since a storage period of more than four months at low temperature (11°C) is considered essential to induce germination of *C. antiqua* cysts recovered from the field (Imai and Itoh, 1987), the germination of laboratory cysts was investigated after cold treatment (9-10°C) for five months (Experiment 7). At the end of the cold treatment, the number of laboratory cysts on the surface of the coverslips was reduced to approximately 10% of the initial levels (*i.e.* from *ca.* 100 to 10 cells cm^{-2}), and ruptured cyst walls were observed on the glass. Furthermore, in the germination experiments, vegetative cells were recovered from incubations from only eight pieces of coverslip among 29 pieces examined. On the basis of the counts of the original cysts before mortality, the germination success can be estimated at $\ll 1\%$.

4. Discussion

Although sexual reproduction of Raphidophycean algae has not yet been reported (*cf.* Subrahmanyan, 1954), the observations——(1) the mean DNA content of cysts was approximately double that of G_1 cells; and (2) 'small' cells fused to become 'triangle'-shaped cells, many of which were transformed into non-motile laboratory cysts——strongly suggest that our laboratory cysts are formed through a sexual process. Induced by P- or N-depleted conditions, gametes ('small cells') are formed that fuse to form planozygotes ('triangle'-shaped cells) which then become hypnozygotes (laboratory cysts). In our crossing experiment, gametes ('small cells') were not formed in P-depleted cultures of strains Ho-1 and B-5. This is not unusual in studies of sexuality of other phytoplankton, since not all isolates of a species respond equally well to induction cues in laboratory cultures (Anderson *et al.*, 1984). The inability of these strains to form gametes is currently under investigation.

In life cycle studies of phytoplankton species, it is important to clarify whether sexual fusion is homo- or heterothallic. Although strong interaction between strains A-0 and B-20 was observed (Table 3), it is difficult to conclude the thallism of *C. antiqua* at present, since some laboratory cysts were also formed when these two strains were each grown as clonal cultures. Two different explanations are offered to explain these results: (1) encystment is heterothallic in this species, and thus the laboratory cysts formed within clonal cultures are asexual products (parthenogenesis); or (2) encystment is homothallic and strong interaction between strains A-0 and B-20 is due to 'selective mating'. Namely, each strains has an ability to form sexual zygotes within clonal cultures, but the incidence of mating

with another clone is higher than that with himself. Although we cannot conclude which explanation is the case, it seems difficult to explain the strong interaction between A-0 and B-20 in terms of parthenogenesis alone. The thallism of *C. antiqua* is being investigated by studies of the DNA content of laboratory cysts formed in clonal cultures.

During cold treatment (9–10°C; Experiment 7), *ca.* 90% of the laboratory cysts died, and the incidence of germination was extremely low ($\ll 1\%$). Storage temperature in the experiment was lower than that experienced by cysts recovered from the Seto Inland Sea (11°C; Imai and Itoh, 1987); this difference may explain the extensive mortality and low germination incidence. Another possibility is that toxic materials were excreted from the glass storage vials, which were not borosilicate glass (Anderson *et al.*, 1984). However, in order to clarify the cause and the time course of the mortality of laboratory cysts, the effects of storage temperature and bottle type should be examined.

Cysts of *C. antiqua* were formed under P- or N-depleted conditions with yields of 50–250 cells cm^{-2}. The incidence of encystment (*CI*) was calculated to be 2–10% based on the following equation:

$$CI = 2\frac{N_c}{N_v \cdot d} \times 100, \qquad (1)$$

where N_c is the cyst yield (cells cm^{-2}), N_v the cell concentration at the growth maximum, d the depth of the medium (*ca.* 2 cm). Here, factor of 2 was introduced to account for the sexual fusion of two cells to make each zygote. One may think that the *CI* value obtained above is rather low. However, similar percentages have been obtained in laboratory encystment studies of dinoflagellates (Anderson *et al.*, 1985; Anderson and Lindquist, 1985).

We can examine the validity of these estimates by calculating the potential cyst yield from natural blooms of *C. antiqua*. If Eq. (1) is applied to *C. antiqua* red tides in 1987, in which the average cell concentration (N_v) over the water column ($d = 2 \times 10^3$cm) was 250 cells ml^{-1} when N-nutrients were exhausted (Nakamura *et al.*, 1989), then we obtain an N_c of $0.5\text{–}2.5 \times 10^4$ cysts cm^{-2}. These values are one or two orders of magnitude higher than the observed abundance of cysts in bottom sediments (20–1,000 cysts cm^{-2}; Imai and Itoh, 1985), which does not appear to change significantly between years (I. Imai, personal communication). It would appear that a 2–10% encystment efficiency is sufficient to explain the actual delivery of cysts to bottom sediments from natural blooms.

In summary, considerable progress has been made in our studies of the life cycle of *C. antiqua*. Cysts produced in laboratory cultures appear to be identical to those from natural sediments. Laboratory experiments on these cysts will tell us much about the dynamics of this important plankton species.

Acknowledgements

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Chattonella antiqua の培養系でのシスト形成

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要旨: 培養系において, *Chattonella antiqua* (ラフィド藻) のシスト形成に初めて成功した。リンないし窒素の枯渇した本種の培養中にカバーグラスを敷くと, シストは, その表面上に形成され, 形成量は 50-250 cysts cm^{-2} であった。交配実験の結果, シストはある特定の2株の組合せで最もよく形成されたが, クローン内でのシスト形成もみとめられた。シスト中の DNA 含量は, 同調的に増殖している栄養細胞の G_1 期での DNA 量の約2倍であった。また, 光学顕微鏡下での観察によると, リン枯渇条件下, 「微小細胞」が形成され, これが細胞融合をおこなって「三角細胞」に変化し, 最終的にシストを形成することが判明した。これらの結果は, シストが有性生殖の結果生じる diploid であることを強く示唆している。そして, 「微小細胞」, 「三角細胞」, 「シスト」はそれぞれ gamete, planozygote, hypnozygote に相当すると考えられた。冷暗条件下 (9°C) で5カ月保存したシストのうち, 約 90% は, 保存中に死滅したが, 生き残ったシストからの発芽がみとめられた。

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